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EFFECT OF NAFAMOSTAT MESILATE, A SYNTHETIC PROTEASE INHIBITOR, ON TISSUE FACTOR-FACTOR VIIa COMPLEX ACTIVITY.

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Abstract Nafamostat mesilate (NM), a synthetic protease inhibitor, is frequently used for the treatment of disseminated intravascular coagulation (DIC) in Japan. NM inhibits several proteases which may be importantly involved in the pathophysiology of DIC. Since tissue factor (TF) plays a critical role in DIC associated with septicemia, inhibition of the extrinsic pathway of coagulation by coagulation inhibitors may be useful for the treatment of DIC. NM inhibited extrinsic pathway activity (TF-F.VIIa mediated-F.Xa generation) in a concentration dependent manner; the IC_{50} was 1.0×10^{-7} M. F.Xa was not inhibited by NM at the concentrations used in the experiment, suggesting that NM might inhibit TF-F.VIIa complex activity. When incubated with TF-F.VIIa complex, NM inhibited the complex activity with an IC_{50} of 1.5×10^{-7} M, the same value that found for inhibition of extrinsic pathway activity. A Lineweaver-Bulk's plot of the inhibition demonstrated that NM inhibited TF-F.VIIa complex in a competitive fashion, with an inhibition constant (Ki) of 2.0×10^{-7} M.

These findings suggested that NM may be a potent inhibitor of TF-F.VIIa complex and the therapeutic effect of NM in DIC patients could be partly explained by inhibition of the extrinsic pathway of the coagulation system.

The blood coagulation system consists of intrinsic and extrinsic pathways; the former is initiated by activation of F.XII on a negatively charged surface, and the latter is initiated by binding of F.VII to tissue factor (TF) which is derived from the injured tissue, activated monocytes or endothelial cells (1). Recent investigations of the blood

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coagulation system have demonstrated that the extrinsic pathway plays a more important role in fibrin formation than the intrinsic pathway (2). Based on this consideration, it is likely that TF plays a critical role in microthrombi formation in the pathophysiology of disseminated intravascular coagulation (DIC). Consistent with this notion is the observation that tissue factor expression in endotoxin- or cytokine-activated monocytes plays a central role in the activation of intravascular coagulation in patients with septicemia (3-5). TF is also expressed on endotoxin- or cytokine-stimulated endothelial cell surfaces and it can activate the extrinsic pathway of coagulation to contribute to microthrombi formation in DIC associated with septicemia (6). Endotoxin administration to normal human subjects resulted in the activation of coagulation without activation of the contact system, suggesting that the extrinsic, but not the intrinsic, pathway plays a key role in the activation of coagulation in septicemia (7). Taken together, these observations further suggested that inhibition of the extrinsic pathway may be important for the treatment of DIC associated with septicemia. Among the physiological protease inhibitors, tissue factor pathway inhibitor (TFPI) is the most potent inhibitor of TF-F.VIIa complex in the presence of F.Xa (8). However, whether TFPI regulates TF-F.VIIa complex activity in the pathophysiology of DIC is uncertain (10). In Japan, nafamostat mesilate (NM), a synthetic protease inhibitor, that inhibits several proteases of the coagulation and fibrinolysis system is frequently used as one of therapeutic agents for DIC. However, little information is available concerning the effects of NM on the extrinsic pathway of the coagulation system.

In the present study, effect of NM on the extrinsic pathway of the coagulation system was investigated to determine whether NM might be useful for the treatment of DIC associated with septicemia.

MATERIALS AND METHODS

Materials

The chromogenic substrate Bzl-Ile-Glu-Gly-Arg-p-nitroanilide (S-2222) and H-D-Ile-Pro-Arg-p-nitroanilide (S-2288) were obtained from Chromogenix (Stockholm, Sweden). TF, F.VIIa and F.Xa were kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Purified TF was mixed with phospholipid vesicles (the molar ratio of protein to phospholipid was 1:100,000) that were composed of phosphatidylserine and phosphatidylcholine in a ratio of 4:6 in the presence of octylglucoside (11). F.VII was not detected in the F.VIIa solution upon SDS-PAGE analysis. F.VIIa preparation had no amidolytic activity toward S-2238 and S-2222, indicating that the purified F.VIIa preparation did not contain thrombin or F.Xa. NM was kindly provided by Torii Pharmaceutical Co. (Tokyo, Japan). Other reagents used were of analytical grade.

Measurement of activities of the extrinsic pathway and TF-F.VIIa complex

TF (20 ng/ml) was incubated with F.VIIa (0.5 μg/ml) for 10 min at 37°C in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl and 5 mM $CaCl_2$ in the presence or absence of various concentrations of NM. F.X (0.75 μg/ml) was then added to the solution and incubated for 10 min at 37°C. The reaction was terminated by the addition of 10 mM of EDTA. Generated F.Xa was measured by the increase in A405 after addition of 2 mM of S-2222, using a spectrophotometer (Beckman DU-64). Activity of TF-F.VIIa complex in the presence or absence of NM were

measured using chromogenic substrate S-2288. Complex formation of TF with F.VIIa was accomplished by incubation of these two compounds for 10 min at 37°C in 50 mM Tris-HCl buffer (pH 7.4) containing 5 mM $CaCl_2$, 0.15 M NaCl and 0.5% bovine serum albumin. After incubation of various concentrations of NM (0~1.0 x 10^{-6} M) with TF-F.VIIa complex in the same buffer for 10 min at 37°C. the remaining TF-F.VIIa complex activity was assayed with 0.4 mM S-2288.

RESULTS

Effect of NM on extrinsic pathway activity

To determine whether NM could inhibit the extrinsic pathway of coagulation, the effect of NM on TF-F.VIIa-mediated Xa generation was examined. As shown in Fig. 1, NM markedly inhibited the activity in a concentration dependent manner. The 50% inhibition concentration (IC_{50}) was calculated at 1.0 x 10^{-7} M. F.Xa activity was not significantly inhibited by NM at concentrations up to 5.0 x 10^{-6} M (Fig. 1). These findings suggested that NM might inhibit the extrinsic pathway of coagulation by inhibiting TF-F.VIIa complex activity.

Effect of NM on the activitiy of TF-F.VIIa complex

To determine the mechanism whereby NM inhibits extrinsic pathway activity, the effects of NM on the activities of TF-F.VIIa complex was examined. NM inhibited

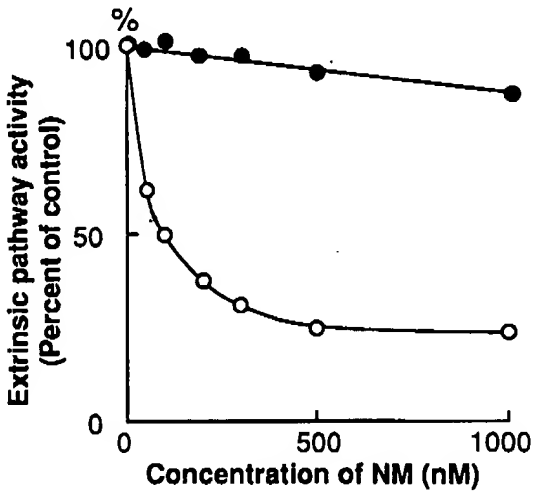

FIG. 1

Effect of NM on F.Xa activity or extrinsic pathway activity of the coagulation system.

Effects of NM on the activities of F.Xa (closed circles) or TF-F.VIIa complex-mediated Xa generation (extrinsic pathway activity) (open circles) were examined. Extrinsic pathway activity was measured as described in Materials and Methods. A control experiment was performed in the absence of NM.

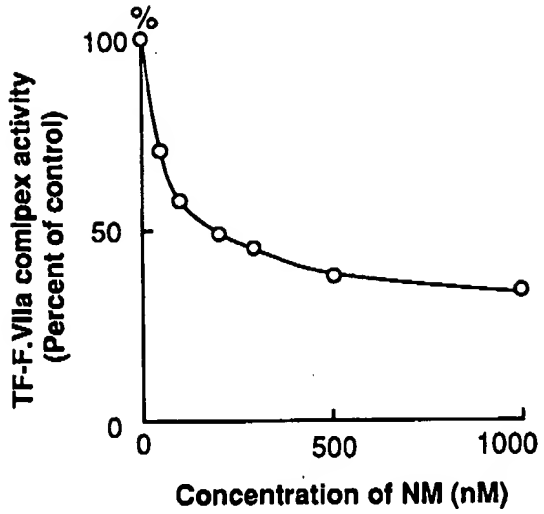

FIG. 2

Effect of NM on the activity of TF-F.VIIa complex.
Effect of NM on the activity of TF-F.VIIa complex was examined. Activity of TF-F.VIIa complex was measured as described in Materials and Methods. A control experiment was performed in the absence of NM.

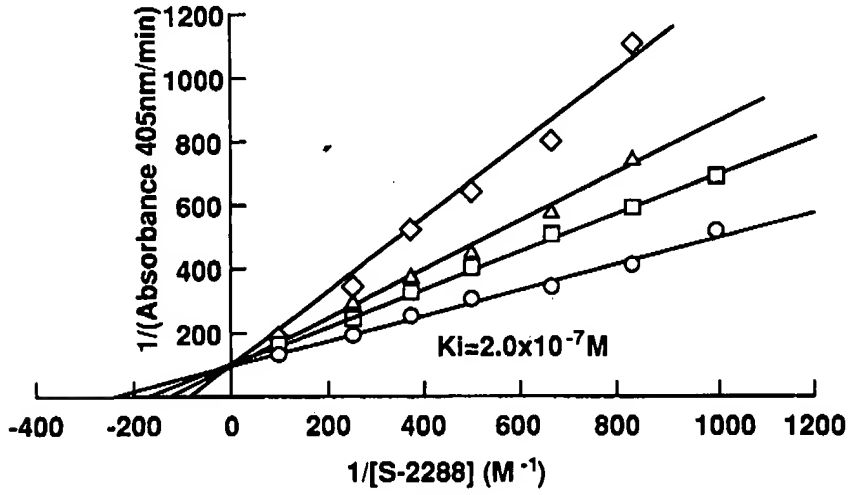

FIG. 3

Lineweaver-Bulk's plots of the inhibition of TF-F.VIIa complex.
Lineweaver-Bulk's plots of the inhibition of TF-F.VIIa complex with or without NM. NM were present at concentrations of 0 (○), 100 (□), 200 (△), and 300 (◇) nM. The inhibition constant (Ki) for TF-F.VIIa complex was 2.0×10^{-7}M.

TF-F.VIIa complex activity in a concentration dependent manner; the IC_{50} for TF-F.VIIa complex was 1.5×10^{-7} M (Fig. 2). A Lineweaver-Bulk's plot demonstrated that NM inhibited TF-F.VIIa complex activity in a competitive fashion, and the inhibition constant (Ki) was 2.0×10^{-7} M (Fig. 3). These findings indicated that NM inhibited the extrinsic pathway activity by inhibiting TF-F.VIIa complex competitively.

DISCUSSION

In the present study, NM was shown to inhibit TF-F.VIIa complex in a competitive fashion, thus inhibiting the activity of the extrinsic pathway of the coagulation system. NM is frequently used for the treatment of DIC in Japan; its efficacy was demonstrated in 70% of DIC patients treated with this agent (12). The IC_{50} of NM was 1.5×10^{-7} M for TF-F.VIIa complex. This concentration of NM was within the plasma concentration range (2.8×10^{-8}-2.4×10^{-7} M) of NM observed after administration of therapeutic doses of NM (0.1-0.2 mg/kg/hr) to patients with DIC (12). Thus, it is possible that NM inhibits the extrinsic pathway of coagulation in patients with DIC. NM was demonstrated to inhibit various proteases that might be importantly involved in the pathophysiology of DIC, such as thrombin, F.Xa and plasmin (13, 14). Since it was demonstrated that TF plays a critical role in the pathophysiology of DIC with septicemia (3,4) and also in non-septic pathologic conditions (5), the clinical effectiveness of NM in the treatment of DIC could be partially explained by the inhibition of the extrinsic pathway of the coagulation system.

Physiological inhibitors for the extrinsic pathway are TFPI (8) and AT III-heparin (15). TFPI potently inhibits TF-F.VIIa complex in the presence of F.Xa at physiological concentration (8) and inhibits TF-F.VIIa complex in the absence of Xa at high concentration (16). Despite the apparent importance of TFPI in the regulation of the blood coagulation system in vitro, the pathophysiological significance of TFPI in DIC has not yet been elucidated. It was demonstrated that TFPI levels were not decreased in patients with DIC (9, 17). Administration of TFPI prevents coagulation response and lethal effect of E.coli injection in baboon, but TFPI level needs to achieve 20-fold increase in normal TFPI serum level (18). Since TFPI inhibits TF-F.VIIa only in the presence of F.Xa at physiological concentration (16), the delay in the inhibition of TF-F.VIIa before sufficient F.Xa is generated to be complexed with TFPI may explain why TFPI could not prevent TF-induced DIC (9).

Recently, Chabbat et al (19) demonstrated that aprotinin was a competitive inhibitor for the TF-F.VIIa complex. Aprotinin inhibited F.VIIa only when it was complexed with TF. The IC_{50} was calculated to be about 3.0×10^{-5} M. The inhibition was about 200 times less potent than that induced by NM.

Although AT III did not inhibit F.VIIa in the absence of heparin (20), it inhibited TF-F.VIIa complex in the presence of heparin ($Ki = 4.4 \times 10^{-7}$) (21,22) or on the endothelial cell surface where glycosaminoglycans (GAGs) are abundantly present (15). However, plasma levels of AT III were markedly decreased in DIC associated with septicemia (23) and endothelial cell surface GAGs were shown to be decreased by the action of endotoxin or cytokines (24). Thus, in such pathological conditions associated with septicemia, NM may be a useful therapeutic agent for the patients with DIC.

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